SYNOPSIS OF THE THESIS SUBMITTED TO THE UNIVERSITY OF NMIMS (DEEMED-TO-BE UNIVERSITY) FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (BIOLOGICAL SCIENCES)

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CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common autoimmune chronic inflammatory disorder [1]. It is considered under musculoskeletal conditions. A study reports the prevalence of musculoskeletal disorders that vary between 7.08–11.5%. The prevalence of RA between countries is estimated to vary from 0.5–1% [2]. Being an autoimmune disorder, it is not curable, thus only the symptoms can be treated and progression of the disease can be delayed. Treatment for RA aims to control the pain, inflammation and to slow the progression of the disease. The treatment regimen includes Disease-modifying antirheumatic drugs (DMARDs), Non-steroidal anti-inflammatory drugs (NSAIDs), Corticosteroids, and analgesics. Biologics (infliximab, etanercept, rituximab, etc.) have also been developed during the last decade of intense research to control the anti-inflammatory agents (TNF alpha). Research is being done on combination therapy where a DMARD is given in combination with other medication such as Biologics (rituximab)/DMARDs (leflunomide)/ NSAIDs (diclofenac)/ Corticosteroids (prednisolone) for treating RA [3–5]. Furthermore, the pathogenesis of RA has revealed the role of oxidative stress and antioxidants. A compromised antioxidant defense system has been reported in RA patients. It has also been observed the anti-inflammatory effect of antioxidants in experimentally induced arthritis rat models. Thus, antioxidants can be considered as a therapy for the treatment of RA [6–8]. Additionally, nanotechnology-based drug delivery can be considered as an alternative approach for treating RA. It is a multidisciplinary approach that employs a diverse array of tools and techniques aimed at diagnosis of the disease and delivery of the therapeutic agents. Drug-loaded nanoparticles, that can be targeted to macrophages have been used as a tool to treat an autoimmune disorder such as RA. Nanoparticles tend to accumulate in the Reticuloendothelial system (RES) such as the spleen and liver. This property is exploited in developing nano-formulation-based drugs for the treatment of RA [9].

Solid Lipid Nanoparticles [10-15]

Solid Lipid Nanoparticles (SLN) have evolved out from the pool of nanoparticles enhanced with potential application in the pharmaceutical field, cosmetics, research, clinical medicine, and other allied science. SLN size ranges from 50–100nm, which can pass through the blood-brain barrier, thus widely being studied for treating brain disorders. SLN is now widely studied as a colloidal drug carrier, an alternative to the conventional colloidal matrix carrier system. They have many advantages such as good biocompatibility, low toxicity, prolonged controlled drug release, excellent tolerability, good stability against particle growth, and drug hydrolysis. Due to its solid matrix, it also prevents leakage of drugs which was the hurdle in the conventional colloidal drug carriers. Another advantage of SLN is its ability to increase the bioavailability of the drug and can be used for any route of administration. SLN formulated drugs are cost-effective during scale-up processes. Another important property of SLNs reported is the carriers themselves possess no acute and chronic toxicity. Thus, serves as an ideal drug delivery system.

Solid Lipid Nanoparticles for treating Rheumatoid Arthritis [16–22]

SLNs possess, properties such as controlled drug release, low toxicity, good biocompatibility, and good stability, they can be used in treating RA to reduce the side effects and improve response. Studies on SLN show its application of drug delivery through various routes. Therefore, SLN as colloidal drug carriers is studied for controlled targeted drug delivery. Studies are being carried out to evaluate the efficiency of SLN to encapsulate DMARDs for the treatment of RA. The results concluded that SLN exhibits sustained drug release, lowers toxicity, and can also serve as a passive targeting agent in RA. In another study utility of SLN for topical drug delivery was studied. The results suggested that SLN can serve as an efficient promoter for the drug to penetrate the skin. RA being a chronic inflammatory disease of the joint synovium, SLN can be loaded with drugs to target the joints where the inflammation is observed and pain persists. SLN is widely applied in the cosmetic field and can be exploited as a transdermal application of the encapsulated drug at the site of pain and inflammation.

CHAPTER 2

RATIONALE AIM AND OBJECTIVES

Rationale

RA is a chronic inflammatory disease of the joints. It is considered an autoimmune disorder where the body fails to distinguish between foreign and self-antigens, thus producing an immune response against its own antigen. Literature study shows that anti-rheumatic drugs are prescribed either as a monotherapy or as a combination therapy (DMARDs with NSAIDs or DMARDS with Biologics), to treat the symptoms and delay the progression of the disease [2]. These drugs however have shown adverse drug reactions. Mild to severe toxicity is also observed in patients, with long-term usage of the drugs. One such drug very commonly prescribed for the treatment of RA is Methotrexate (MTX) a synthetic analogue of folic acid considers as a DMARD. Depending on the severity of the disease, MTX is prescribed at lower doses ranging from 2.5–25 mg/week for the treatment of psoriasis and RA. Despite its efficacy, MTX has a low pharmacokinetic profile due to poor solubility, dose-dependent toxicity, shorter half-life, and cellular efflux which limits its use. Regardless of these limitations, MTX is still considered a first-line treatment for RA. However, long-term usage of the drug can cause adverse effects such as mucosal ulceration, stomatitis, pneumonitis, bone marrow suppression, loss of appetite, drug-induced hepatic fibrosis, and cirrhosis and ultimately leading to discontinuation of the medicine. Detailed pathology of RA indicates over-expression of CD44 marker on the surface of synovial lymphocytes, macrophages, and fibroblasts at inflamed joints. In fact, CD44 facilitates the migration of inflammatory cells and activation of various effector cells in the course of RA. Hence this phenomenon can be used in drug targeting wherein hyaluronic acid (HA), a naturally charged biocompatible polysaccharide can be used as a ligand to effectively bind to CD44 markers overexpressed at the site of inflammation.

Further studies on the pathogenesis of RA, have explained the role of oxidative stress and antioxidant. ROS and nitric oxide (NO) are involved in the pathogenesis of RA apart from the inflammatory mediators. Several studies provide evidence of a compromised antioxidant defense system to regulate oxidative stress. Whereas, clinical trials have reported that when the drugs are prescribed as monotherapy or as combination therapy, supplemented with antioxidants have shown better response. Recent studies have also

reported the anti-inflammatory effect of antioxidants (curcumin, Caffeic acid, quercetin (QE)) in experimentally induced arthritis in rats. Thus, antioxidants can be considered as a therapy for the treatment of RA.

Solid Lipid Nanoparticles are made up of a solid hydrophobic core which is a monolayer of phospholipid that remains solid at room temperature as well as body temperature. This property of SLN makes it a suitable carrier for lipophilic drugs. SLN combines the advantages of a traditional carrier system (colloids, polymers, emulsions liposomes) and outgrows their major disadvantages. SLN is now being exploited as a drug carrier for the different routes of administration. SLN because of its small size (100–200 nm) bypasses the liver and spleen metabolism and is directly absorbed from the intestines via the lymphatic system. This enhances the bioavailability of the drugs. SLN also prevents drug leakage and provides controlled drug release. SLN formulated drugs are cost-effective during scale-up processes. Another major advantage of SLN carrier is it's reported to have no acute and chronic toxicity. Thus, serves as an ideal drug delivery system.

Our study aims to synthesize an SLN and to encapsulate in them a DMARD (MTX) and an antioxidant (QE). The purpose of using SLN as a carrier system in our study is to explore its potential for enhanced bioavailability, prolonged drug release, biocompatibility and avoiding drug leakage, and reducing drug toxicity. Additionally, the reason for using an antioxidant (QE) is to enhance the treatment regimen of RA as well as reduce the toxicity and side effects associated with MTX.

Aim

This study aims to explore Solid Lipid Nanoparticle as a drug carrier for the treatment of Rheumatoid Arthritis.

Objectives

- Pre-formulation studies (Drug-drug and Drug-excipient compatibility studies)
- Synthesis and characterization of the synthesized formulations
- Pharmacokinetic studies and acute oral toxicity of the formulations
- *In vivo* Efficacy studies of the drug-loaded SLN formulation.

CHAPTER 3

PRE-FORMULATION STUDIES

This chapter includes an analysis of the complex interactions between MTX and QE with the help of various analytical tools such as Fourier-Transform Infrared (FTIR) spectroscopy, Differential Scanning Calorimetry (DSC), and X-Ray Diffraction Analysis (XRD). Molecular modeling studies were performed to gauge the level of interaction between MTX and QE on the molecular level. The combination was further scanned for any physicochemical interactions using UV–Visible spectroscopy and High-Performance Liquid Chromatography (HPLC). No significant physicochemical interaction was observed from the extensive analytical studies indicating that MTX and antioxidant QE could be a good choice for combination therapy. The results presented herein could be used for developing formulations either incorporating both entities in a single delivery system or administered as a combination therapy to reduce MTX dose, possibly reducing the toxicity associated with it.

Results

FTIR: The spectra of the 1:1 physical mixture showed (figure 1) a few significant peaks of both the drugs as well as overlapping several bands (Figure 2). Since MTX has both –NH as well as –COOH groups while QE has –OH groups; there could be a possibility of the presence of hydrogen bonds and Van der waals attractions that might have caused a slight change in the spectra of the combination drugs.

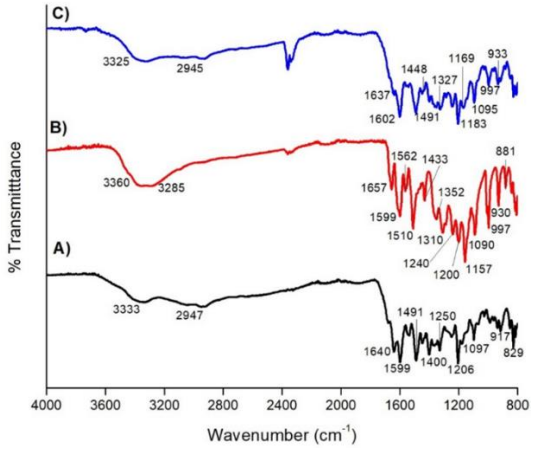

Figure 3.1: FTIR spectra of A) MTX, B) QE, and C) MQ (1:1 physical mixture of MTX and QE).

DSC: DSC scans (Figure 2) of the individual drugs indicate their crystalline structure as well as the endothermic peak at 140°C and 325 °C corresponding to the melting point of

MTX and QE, respectively. However, the 1:1 combination of the drug (figure 3 c) shows a shift in the endothermic peak to 120°C. Once again indicating the possibility of any hydrogen bond interaction between the polyphenol groups of QE with NH groups present in MTX.

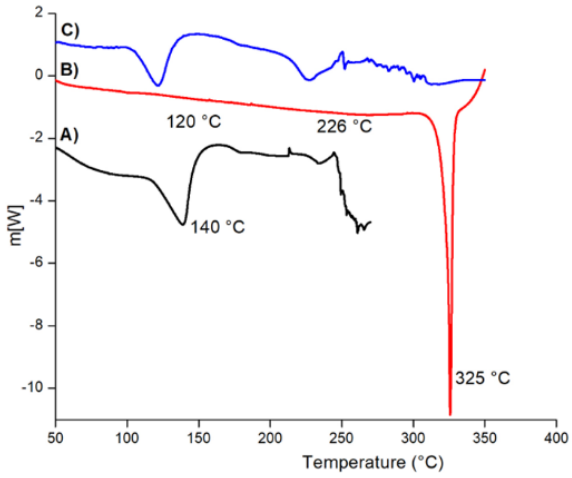

Figure 3.2: DSC thermogram of showing endothermic peak corresponding to the drug melting point a) MTX (140 °C), b) QE (320 °C) and c) 1:1 Combination drug (MQ)

XRD: Both MTX and QE have shown distant peaks indicating the crystalline nature of the pure drugs. However, the 1:1 physical mixture of MTX and QE presented several distinct peaks, indicating crystalline nature (Figure 3). Despite this, the nature of the interaction was not clearly understood for which molecular dynamics simulation study was performed.

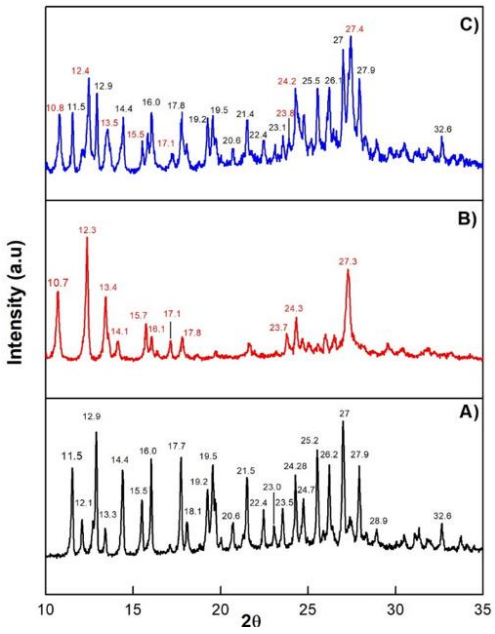

Figure 3.3: X-Ray Diffraction pattern (XRD) of MTX, QE, and 1:1 mixture of the drugs (MQ) showing its 2θ values corresponding to the individual drugs

Molecular Modelling: From Figure 4, it can be clearly observed the nitrogen of the pteridine ring in MTX and the hydroxyl group of QE molecules simulating ligand–ligand interaction via H–bond interaction. Overall, MTX molecules were found to interact with QE simulating the physical interaction *via* electrostatic and H–bonding interactions.

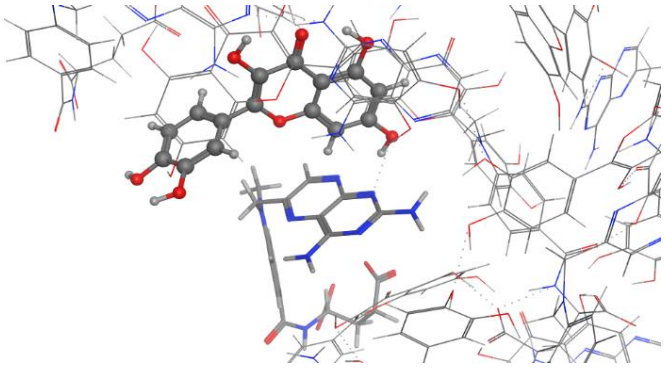

Figure 3.4: Molecular 3D representation of the binding mode of MTX and QE interaction (grey dotted-lines indicate H-bonding interactions). The MTX is shown as a ball-and-stick model while the QE is shown as line models colored by the elements.

UV-Visible Spectroscopy: The individual drugs show lambda max at 306 nm for MTX and 365 nm for QE in the UV–Visible spectroscopy studies. While the combination of the drugs in a 1:1 ratio showed a bell shape curve at 306 nm corresponding to the lambda max of MTX and 355 nm the corresponding to abosrpotion by QE. However, a non-significant shift in the lambda max for QE from 365–355 nm can be observed (figure 5). Thus, it can be clearly understood that no interaction has occurred between MTX and QE. The H–bonding interactions and electrostatic attraction which were observed in the physical mixture by FTIR spectra DSC thermograms, XRD patterns, and MD simulation might have broken down due to the presence of solvents.

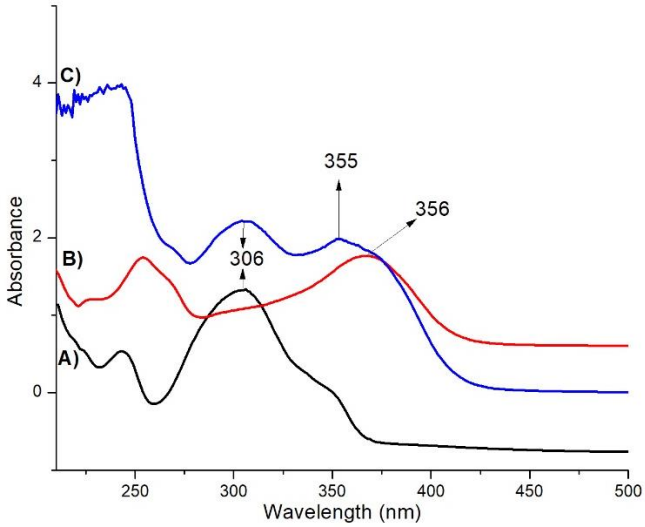

Figure 3.9. UV-vis absorption spectra of A) MTX (black line) B) QE (red line), and the C) MQ (1:1 physical mixture of MTX and QE; blue line).

- **HPLC:** The HPC data (figure 6) shows two distinct peaks for MTX with retention time at 3.3 min and QE with retention time at 5.5 min respectively. This data also coincides with result obtained from UV-Visible spectroscopy that no chemical interaction has occurred between MTX-QE.

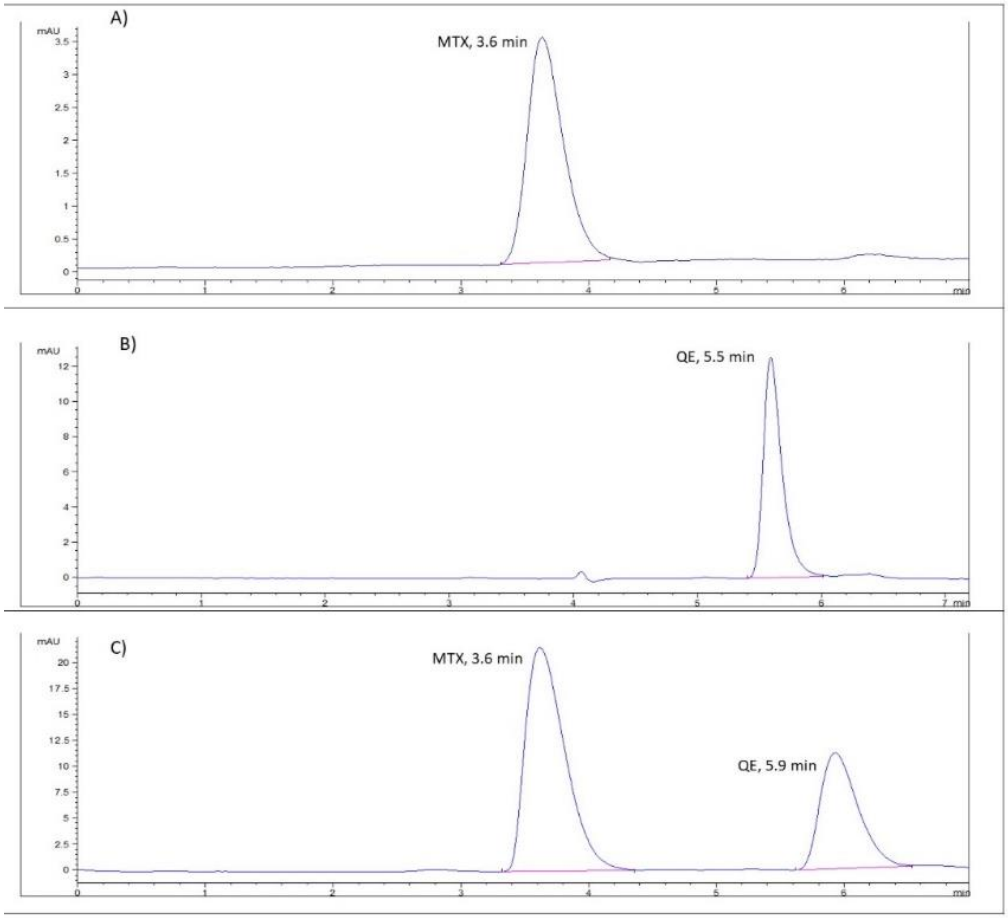

Figure 6: HPLC chromatogram of a) MTX with a retention time of 3.3 min; b) QE with a retention time of 5.5 min; and c) physical mixture of MTX and QE (MQ) (solubilized in acidified methanol showing) with retention time of 3.6 and 5.9 min, respectively.

CHAPTER 4
SYNTHESIS AND CHARACTERIZATION OF SLNs

This chapter deals with the selection of lipid and surfactants based on drug solubility, followed by optimization using the Quality by Design approach (QbD). Later the SLNs were prepared using the High-speed homogenization method and coated by hyaluronic acid (HA) for site-specific drug delivery. The SLN dispersions were spray-dried and finally characterized for various properties. Blank-SLNs (SLNs without any drug), MTX-SLNs, HA-MTX-SLNs, QE-SLNs, and HA-QE-SLNs were prepared and characterized for their size morphology entrapment efficiency, drug release, and crystalline nature.

- **QbD Approach**

  Pharmaceutical QbD is a systemic approach for the development of a product with predefined objectives, emphasizing process understanding and control parameters based on quality risk management. For the optimization of MTX-SLNs and QE-SLNs, Box Behnken design (BBD) was chosen, and further analysis was done using Design-Expert Software (Design-Expert®11). The design is useful in understanding the interaction between the factors if any

  Results for HA-MTX-SLNs: Critical factors affecting the size, PDI and %EE were identified from the 3D graphs represented in figure 4.1 and the final formulation was optimized using the overlay plot (figure 4.2)**.**

  Results for HA-QE-SLNs: Similarly for HA-QE-SLNs the significant factors affecting the size, PDI and %EE were identified from the 3D graphs represented in figure 4.3 and the final formulation was optimized using the overlay plot (figure 4.4)**.**

Table 1: Selection of variables and their levels for MTX-SLNs

Symbol	Variable	Units	-1 level	0 level	+1 level
X1	Amount of lipid	mg	100	300	500
X2	Amount of CTAB	mg	10	30	50
X3	High speed homogenization (HSH) speed	rpm	15,000	17,500	20,000
Dependent Variables (response)					
Y1: Size (nm) Y2: PDI Y3: Percentage Entrapment Efficiency (%EE)					

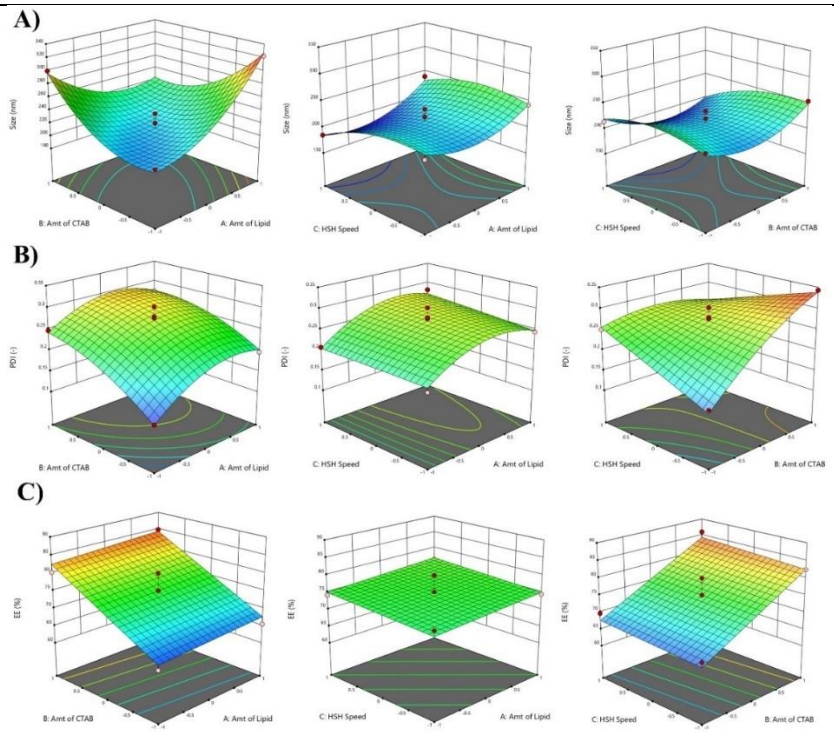

Figure 4.1. 3D response surface plot showing the effect of variables on particle size (A), PDI (B), and %EE (C) for HA-MTX-SLNs

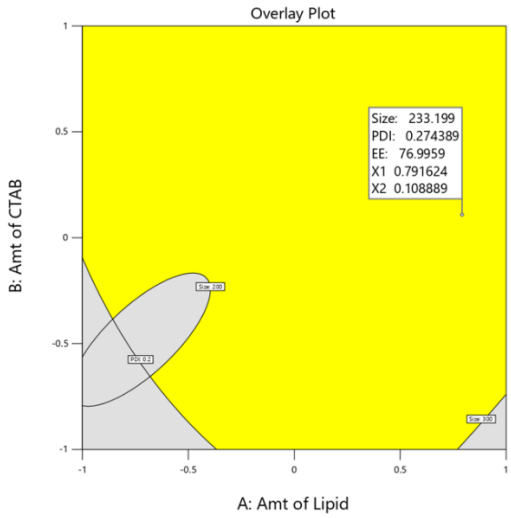

Figure 10: Overlay plot of optimized formulation HA-MTX-SLNs

Table 2: Selection of variables and their levels for QE-SLNs

Symbol	Variable	Units	-1 level	0 level	+1 level
X1	Lipid:Drug: PEG	-	400:10:200	450:10:250	500:10:300
X2	Concentration of Surfactant	%	1	1.25	1.5
X3	High speed homogenization (HSH) speed	rpm	12,000	13,000	15,000
Dependent Variables (response)					
Y1: Size (nm) Y2: PDI Y3: Percentage Entrapment Efficiency (%EE)					

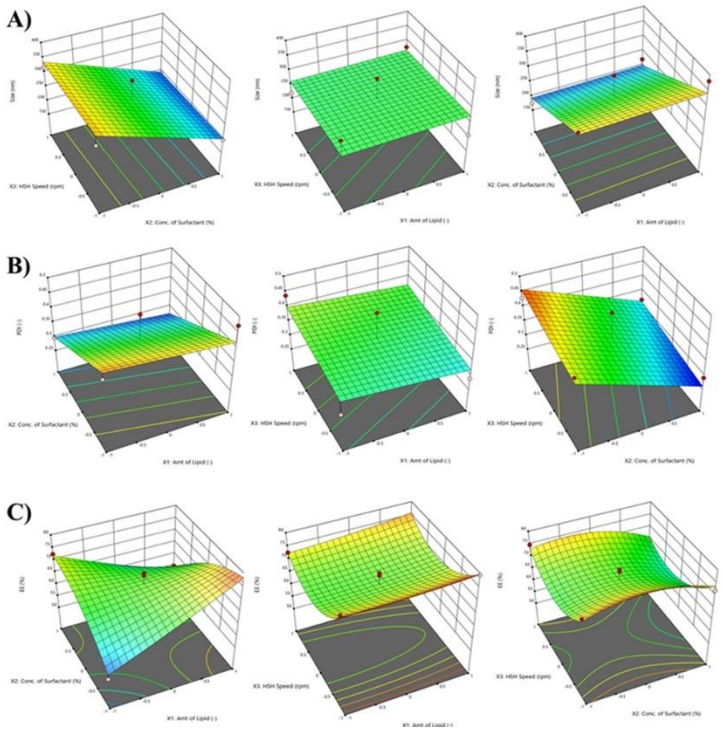

Figure 4.3: 3D response surface plot showing the effect of variables on particle size (A), PDI (B), and %EE (C) for HA-QE-SLNs.

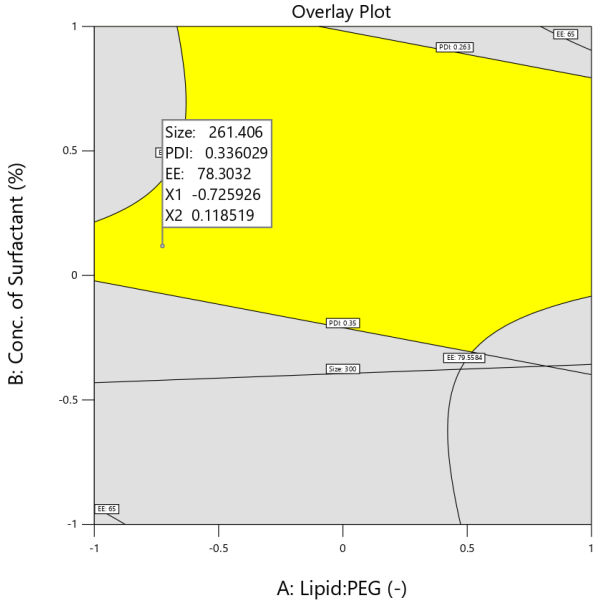

Figure 4.4: Overlay plot of the optimized HA-QE-SLNs.

- **Characterization of HA-MTX-SLNs and HA-QE-SLNs**
  - **Size, PDI, Zeta Potential, and Entrapment efficiency:** Table 3 below provides the size, PDI, entrapment efficiency and zeta potential of the developed formulations.

Table 3: Size PDI, entrapment efficiency and zeta potential of blank SLNs, MTX-SLNs, HA-MTX-SLNs, QE-SLNs and HA-QE-SLNs.

Formulation	Properties			
	Size	PDI	Entrapment Efficiency	Zeta Potential
Blank SLNs	297.9	0.294	-	-10.2
MTX-SLNs	205	0.279	72.88	25.5
HA-MTX-SLNs	239	0.264	78.78	-29.8
QE-SLNs	233	0.232	72.42	-25.8
HA-QE-SLNs	230	0.341	71.9	-41.1

- **TEM analysis:** The surface morphology of the HA-MTX-SLNs and HA-QE-SLNs was analyzed using TEM. The TEM images shown below in figure 15 indicate that both the formulations are spherical in shape. The particle size of HA-MTX-SLNs determined by TEM image analysis using Image J software (Image J 1.53e), was found to be 230 ± 46.4 nm for HA-MTX-SLNs, 280 ± 29.19 nm for HA-QE-SLNs nm which correlated with the size obtained by the zeta sizer.

A **B**

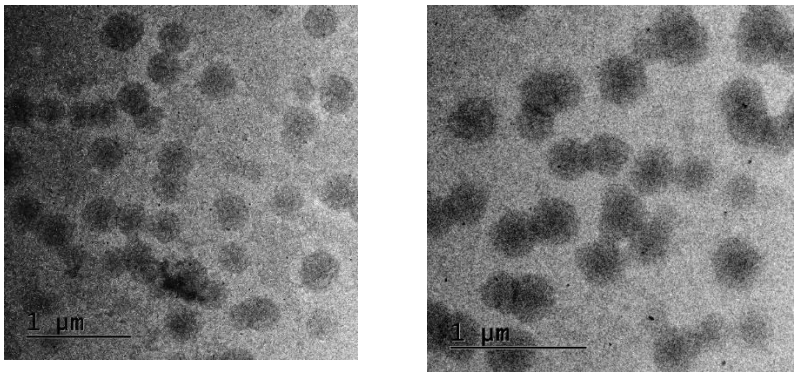

Figure 4.5: TEM images of A) HA-MTX-SLNs, and B) HA-QE-SLNs.

DSC: The DSC thermograms (figure 16) of developed formulations showed endothermic peak only at 160°C corresponding to that of mannitol but no peaks of MTX/QE and/or HA indicating entrapment of drug MTX/QE within the SLNs; protective coating of the SLNs with Mannitol to prevent damage to SLNs from high temperatures during the spray drying process. The absence of a glass transition peak and the presence of a sharp endothermic peak connotes the crystalline nature of SLNs.

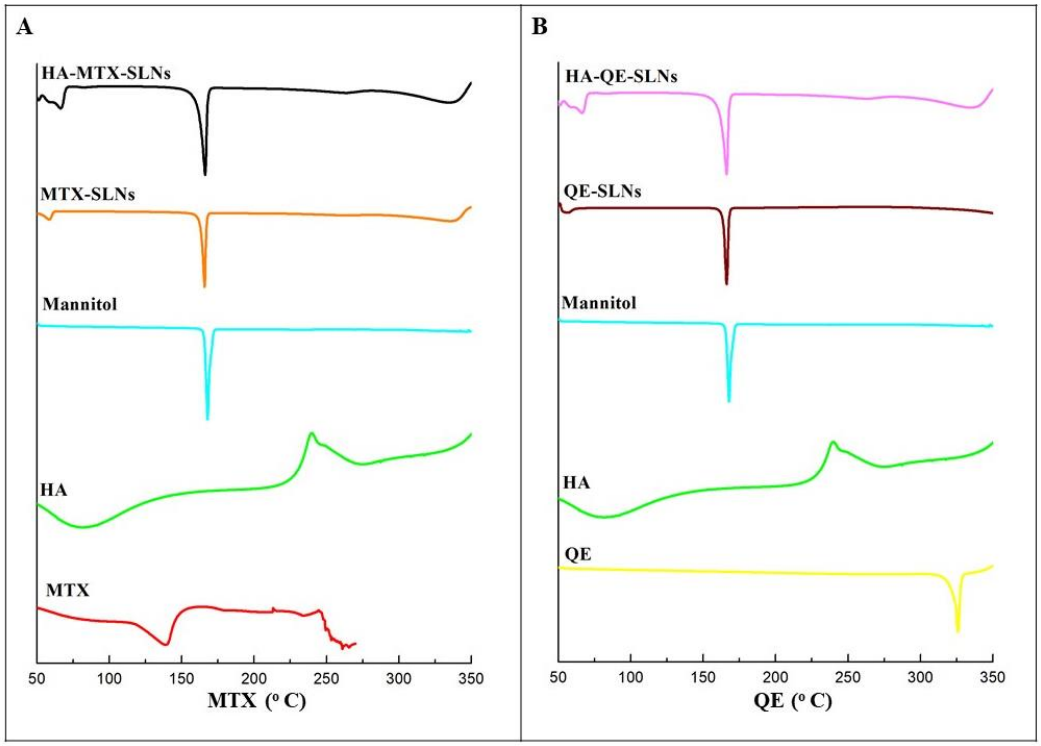

Figure 4.6: DSC thermograms of A) MTX , MTX loaded SLNs; B) QE, QE loaded SLNs.

XRD: XRD pattern (figure 17) of both the developed formulations showed the presence of standard drug as well as mannitol. However, the broadening of peak HA-coated SLNs can be attributed due to the influence of HA, since HA is amorphous in nature and does not show a distinct characteristic peak. Reduced peak intensity and broadening of the peak also indicate that the lipid matrix has polymorphic changes resulting in less ordered crystalline lattice to develop space for embedding more.

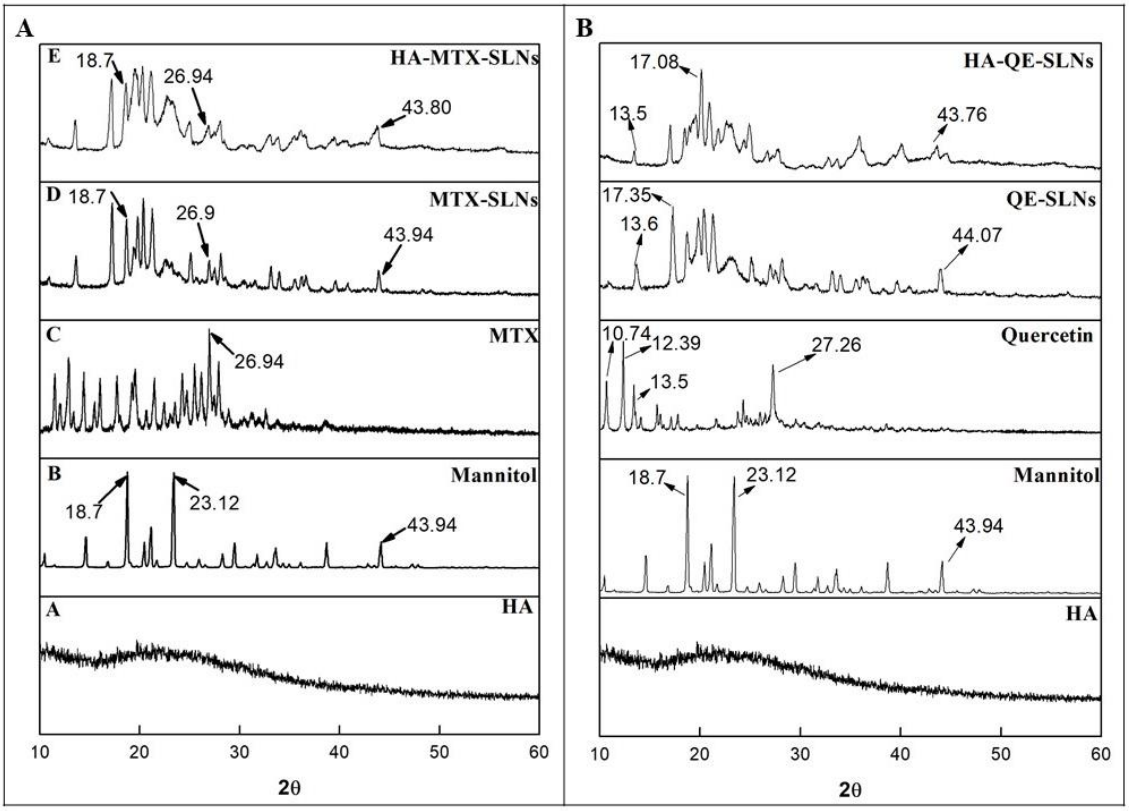

Figure 4.7: X-ray diffraction pattern of A) MTX, MTX loaded SLNs, ad B) QE, QE loaded SLNs.

***In vitro* drug release:** Both the developed formulations have shown (figure 18) sustained drug release which lasted upto 48h. the cumulative drug release data was inserted in various mathematical formulations to determine the mechanism of drug release. Korsmeyers-Peppas model was best suited for both the formulations which indicate that both the drug release pattern shows a concentration-dependent non-fickian diffusion, hence providing sustained drug release.

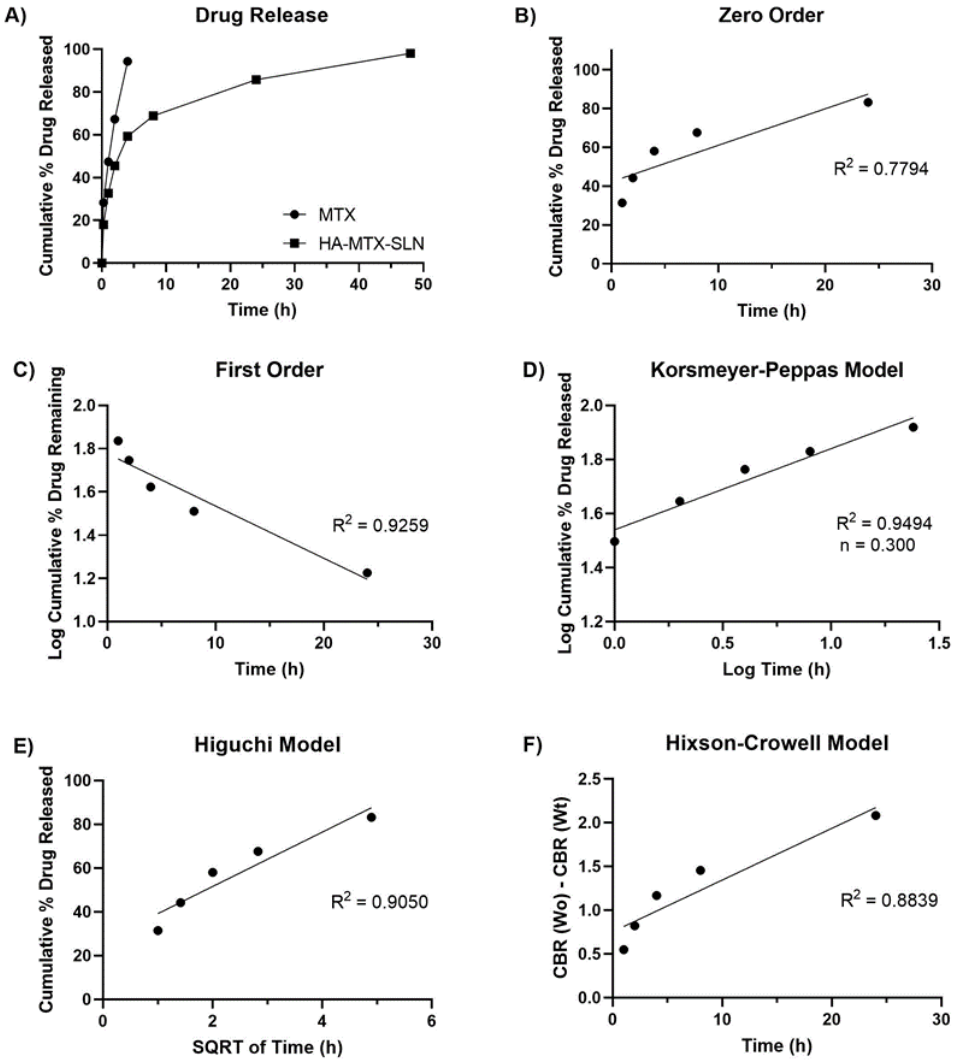

Figure 4.8. *In vitro* release profile of MTX from MTX solution and HA-MTX-SLNs (A) and the release data fitted into various kinetic models (B–F).

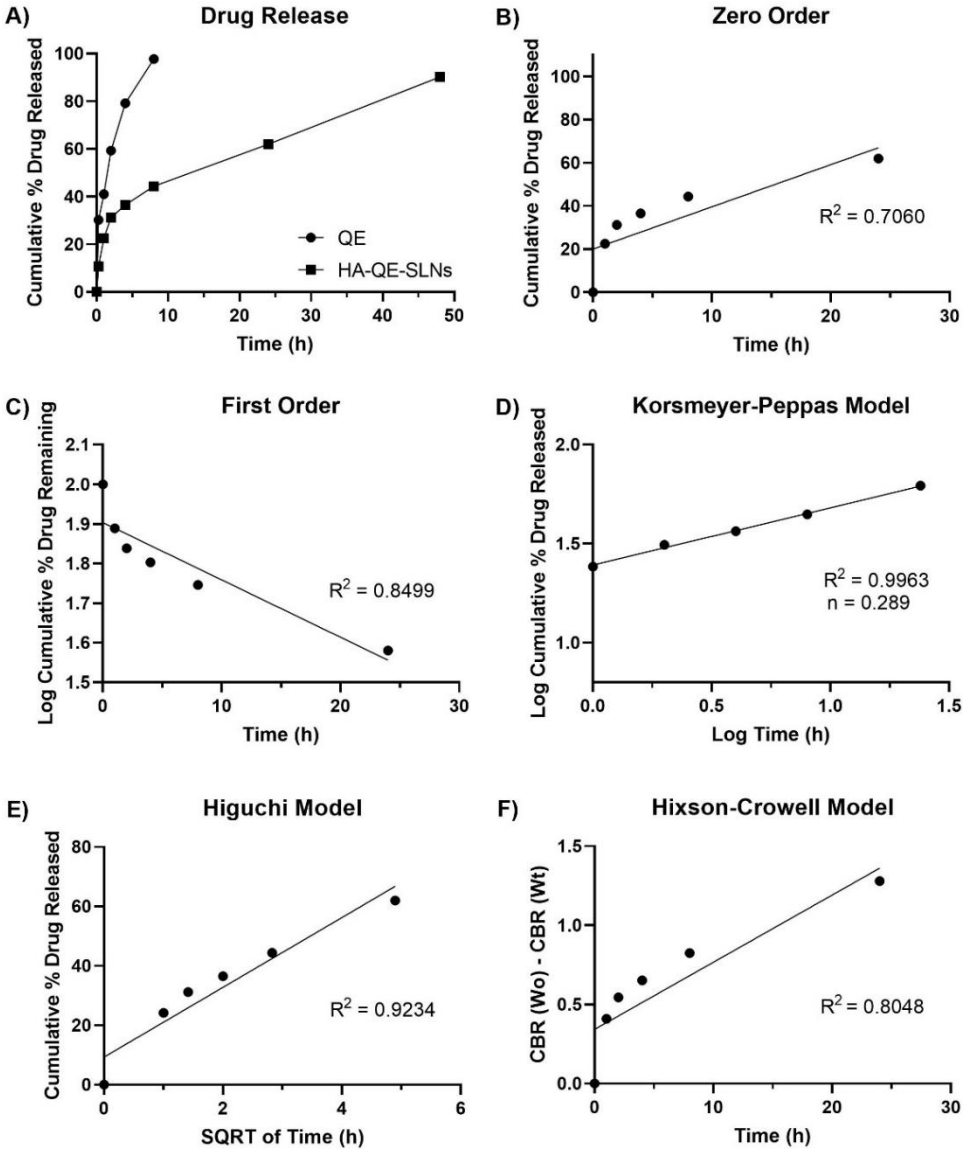

Figure 4.9. *In vitro* release profile of QE from QE solution and HA-QE-SLNs (A) and the release data fitted into various kinetic models (B–F).

CHAPTER 5
ACUTE ORAL TOXICITY AND PHARMACOKINETIC STUDIES AND OF THE DEVELOPED FORMULATIONS

This chapter encompasses toxicity data and the pharmacokinetic profile of the developed formulation. The optimized formulation post characterization was investigated for its acute oral toxicity (AOT) as well as pharmacokinetic (PK) parameters. Toxicity studies were performed using the AOT 425 up and down procedure described in the OECD guidelines in female Wistar rats. AOT 425 software was used to decide the dose to be administered in each rat based on the LD50 value of the standard drug. The software was also used to determine the approximate LD50 value for the developed formulation. Following the toxicity study, PK parameters of the standard drug as well as of the developed formulations were analysed in male Wistar rats. The animals were orally administered with the standard drug and developed formulation. Later the animals were anesthetized and 0.5mL blood was withdrawn for the retro-orbital vein at specific time intervals. The drug was extracted from plasma samples using methanol as an extraction solvent. Drug concentration in the plasma samples was determined by HPLC. The PK parameters were analyzed using PKSolver.

Results

Acute oral toxicity

The optimized formulation post characterization was investigated for its acute oral toxicity (AOT) as well as pharmacokinetic (PK) parameters. Toxicity studies were performed using the AOT 425 up and down procedure described in the OECD guidelines in female Wistar rats. Both HA-MTX-SLNs, and HA-QE-SLNs were found to be safe following the limit test when administered orally at a dose of 2000 mg/kg.

PK parameters

The various PK parameter determined using the PKsolver is listed in table below. The data enunciated that HA coated SLNs have improvised the pharmacokinetic profile of the drug.

Table 5: PK parameters of MTX; HA-MTX-SLNs; QE; and, HA-QE-SLNs determined using PK solver.

Parameters	Samples			
	MTX (1 mg/kg)	**HA-MTX-SLNs (1 mg/kg as MTX)**	**QE (30 mg/kg)**	**HA-QE-SLNs (30 mg/kg as QE)**
AUC_{0-t} (µg·h\mL)	100.71 ± 10.94	101.27 ± 3.76	7513.97 ± 254.44	9939.38 ± 489.62
$AUC_{0-\infty}$ (µg·h\mL)	107.35 ± 11.68	125.01 ± 4.65	28946.20 ± 538.94	47556.95 ± 2341.71
$T_{1/2}$ (h)	5.86 ± 0.63	10.09 ± 0.50	5.11 ±0.09	11.07 ± 0.57
C_{max} (µg/mL)	11.02 ± 0.37	8.83 ± 0.75	375.00 ± 14.88	462.68 ± 13.11
T_{max} (h)	2	4	4 ± 0.07	8 ± 0.39
% Relative bioavailability	–	116.45	-	164.29

CHAPTER 6

In Vivo EFFICACY OF THE DEVELOPED FORMULATION

In this chapter, the therapeutic efficacy of the developed formulations performed in vivo is discussed. The efficacy study was performed on male Wistar rats induced with 0.1 ml complete Freund's adjuvant (CFA) in the left hind paw and treated at different dose levels of the developed formulations individually as well as in combination. Parameters like reduction in paw inflammation, levels of cytokines, oxidative stress markers were measured. Histopathology of the vital organs like liver ,kidney, spleen and paw was also performed to check for any abnormalities. Radiographic analysis of the paw was also carried out to gr

Table 6.1. Treatment groups for efficacy study of HA-MTX-SLNs and HA-QE-SLNs individually in CFA rat model

Group	Treatment	
	MTX-based Formulations	**QE-based Formulations**
1	Normal Control – Saline	
2	Arthritic control – Saline	
3	CMC (0.5%)	
4	Blank-SLNs (CTAB)	Blank-SLNs (PEG)
5	Standard MTX (0.1 mg/kg)	Standard QE (30 mg/kg)
6	Standard MTX (1 mg/kg)	QE-SLNs (≡ 30 mg/kg of QE)
7	MTX-SLN (≡ 1 mg/kg of MTX)	HA-QE-SLNs (≡ 15 mg/kg of QE)
8	HA-MTX-SLN (≡ 0.1 mg/kg of MTX)	HA-QE-SLNs (≡ 30 mg/kg of QE)
9	HA-MTX-SLN (≡ 1 mg/kg of MTX)	

Table 6.2. Treatment groups for efficacy study of combination of HA-MTX-SLNs and HA-QE-SLNs in CFA rat model

Group	Treatment
1	Normal Control – Saline
2	Arthritic control – Saline
3	CMC (0.5%)

4	Blank-SLNs (CTAB) + Blank-SLNs (PEG)
5	Standard MTX (1 mg/kg) + Standard QE (30 mg/kg): MQ
6	HA-MTX-SLNs (≡ 1 mg/kg of MTX) + HA-QE-SLNs (≡ 30 mg/kg of QE): SMQ-I
7	HA-MTX-SLNs (≡ 1 mg/kg of MTX) + HA-QE-SLNs (≡ 15 mg/kg of QE): SMQ-II
8	HA-MTX-SLNs (≡ 0.1 mg/kg of MTX) + HA-QE-SLNs (≡ 30 mg/kg of QE): SMQ-III
9	HA-MTX-SLNs (≡ 0.1 mg/kg of MTX) + HA-QE-SLNs (≡ 15 mg/kg of QE): SMQ-IV

Results

Post CFA injection, increase in paw inflammation on day one for each animal was considered as 100%. Post treatment the reduction of inflammation in each animal was recorded. All the data was fed into Graph pad Prism software (9.0) and Dunnett's multiple comparison test was applied to determine the p values and level of significance for each group when compared to arthritic control group.

Effect of MTX, MTX-SLNs and HA-MTX-SLNs treatment:

From figure 6.1 it can be observed that all the groups showed a significant reduction in the inflammation from day 1 to day 21 including the arthritic control and Blank SLNs treatment group. The reduction in inflammation in animals in the arthritic control group and Blank-SLNs group can be attributed to body's natural immunity. The animal treated with MTX at the dose of 0.1 mg/Kg, showed a significant reduction in paw edema however, at dose of 1 mg/Kg, MTX showed toxicity and resulted in the death of all the animals in Group 5 between days 8 and 10. A significant reduction was also observed in animals belonging to the MTX-SLNs treatment group, whereas animals treated with HA-MTX-SLNs showed maximum reduction in paw inflammation (91.36±8.15%, $p<0.001$), indicating successful targeted drug delivery by using HA coating.

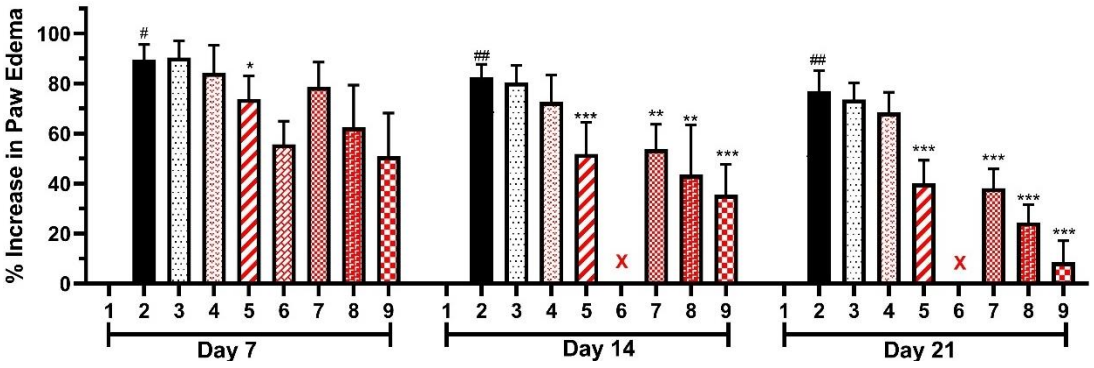

Figure 6.1. Effect of MTX-based formulations on percentage increase in paw edema in CFA-induced rats.

1) Normal control; 2) Arthritic control; 3) 0.5% CMC; 4) Blank-SLNs; 5) standard MTX (0.1 mg/kg); 6) standard MTX (1 mg/kg); 7) MTX-SLNs (equivalent to 1 mg/kg of MTX); 8) HA-MTX-SLNs (equivalent to 0.1 mg/kg of MTX); 9) HA-MTX-SLNs (equivalent to 1 mg/kg of MTX). The values are expressed as mean ± SD. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ were considered significant when compared to the arthritic control group on day 1, whereas $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ were considered significant when compared to the arthritic control groups on the respective days.

Effect of QE, QE-SLNs and HA-QE-SLNs treatment:

Quercetin being an anti-oxidant have shown significant reduction in paw edema indicating its potential anti-inflammatory activity as well (figure 20). Whereas QE-SLNs treatment group has also shown remarkable reduction in paw edema, when compared to standard quercetin treatment, which indicated SLNs enhanced the therapeutic efficiency of the drug. Maximum reduction in the paw edema was observed in animals treated with HA-QE-SLNs at dose equivalent to 30 mg of standard quercetin, highlighting the importance of HA coating for drug targeting.

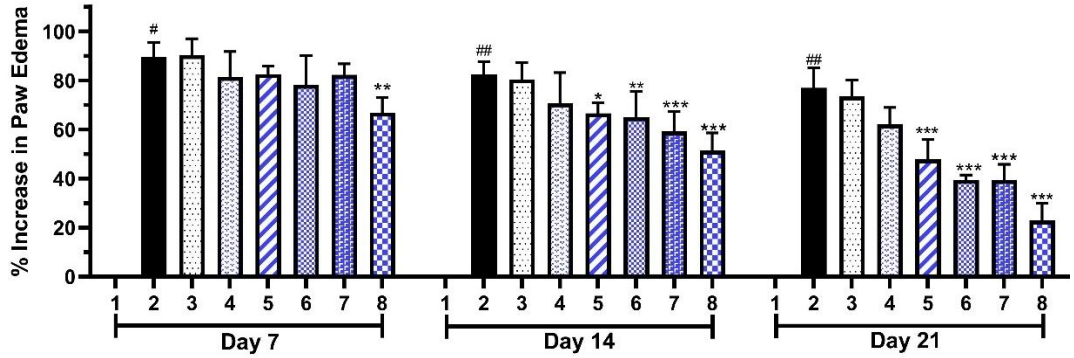

Figure 6.2. Effect of QE-based formulations on percentage increase in paw edema in CFA-induced rats.

1) Normal control; 2) Arthritic control; 3) 0.5% CMC; 4) Blank-SLNs; 5) QE standard (30 mg/kg); 6) QE-SLNs (equivalent to 30 mg/kg of QE); 7) HA-QE-SLNs (equivalent to 15 mg/kg of QE); 8) HA-QE-SLNs (equivalent to 30 mg/kg of QE). The values are expressed as mean ± SD. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ were considered significant when compared to the arthritic control group on day 1, whereas $*p<0.05$, $**p<0.01$, and $***p<0.001$ were considered significant when compared to the arthritic control groups on the respective days.

Effect of combination therapy

Figure 21 exhibit the effect of co-administration of the two drugs on CFA animals. It can be observed that QE when administered with MTX could not significantly reduce the toxicity associated with MTX and hence all the animals died within 14 days of the treatment. However, no such observations were seen in animals treated with SLNs combinations. In fact, combination SMQ1 (HA-MTX-SLNs (equivalent to 1mg of MTX) + HA-QE-SLNs (equivalent to 30 mg of QE)) showed maximum reduction in paw inflammation. However, when compared to the individual HA-MTX-SLNs and HA-QE-SLNs treatment groups the reduction in paw edema was not significant. This could be due some interacting occurring between the two drug at physiological level leading to antagonism effect.

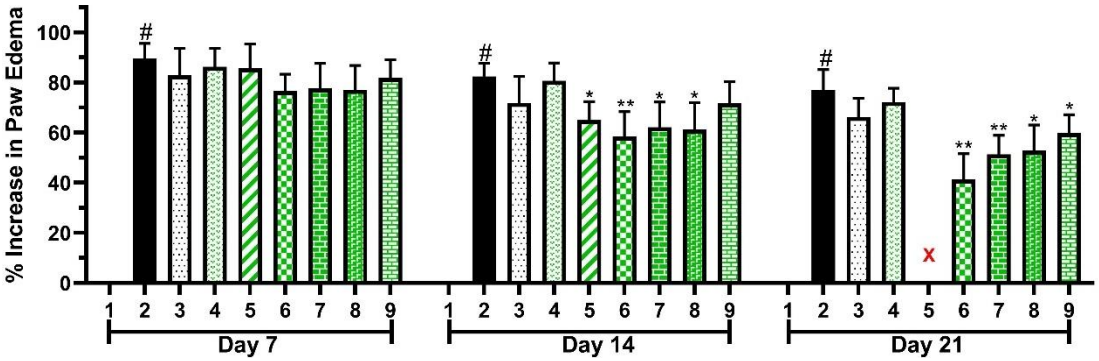

Figure 6.3. Effect of the combination therapy on percentage increase in paw edema in CFA-induced rats.

1) Normal control; 2) Arthritic control; 3) 0.5% CMC; 4) Blank-SLNs; 5) MQ (1 mg/kg of MTX + 30 mg/kg of QE); 6) SMQ-I (equivalent to 1 mg/kg of MTX + 30 mg/kg of QE); 7) SMQ-II (equivalent to 1 mg/kg of MTX + 15 mg/kg of QE); 8) SMQ-III (equivalent to 0.1 mg/kg of MTX + 30 mg/kg of QE); 9) SMQ-IV (equivalent to 0.1 mg/kg of MTX + 15 mg/kg of QE). The values are expressed as mean ± SD. $^{\#}p<0.05$ were

considered significant when compared to the arthritic control group on day 1, whereas *p<0.05 and **p<0.01 were considered significant when compared to the arthritic control groups on the respective days.

Levels of cytokine levels: TNF-α and IL-1β

Levels of inflammatory markers such as TNF-α and IL-1β were measured in the serum samples of animals given treatment as described in the table. Increased levels TNF- α and IL-1β were seen in animals belonging to the arthritic control group and blank SLNs treated group. Whereas significant reductions in TNF-α levels were observed in all other treatment groups. Additionally, a remarkable reduction in the IL-1β levels were observed in animals treated with HA-MTX-SLNs and HA-QE-SLNs individually. HA-MTX-SLNs could bring back the IL-1β levels back to normal. Figure 6.4, 6.5, and 6.6 represent the levels of cytokines measured in CFA-induced animals.

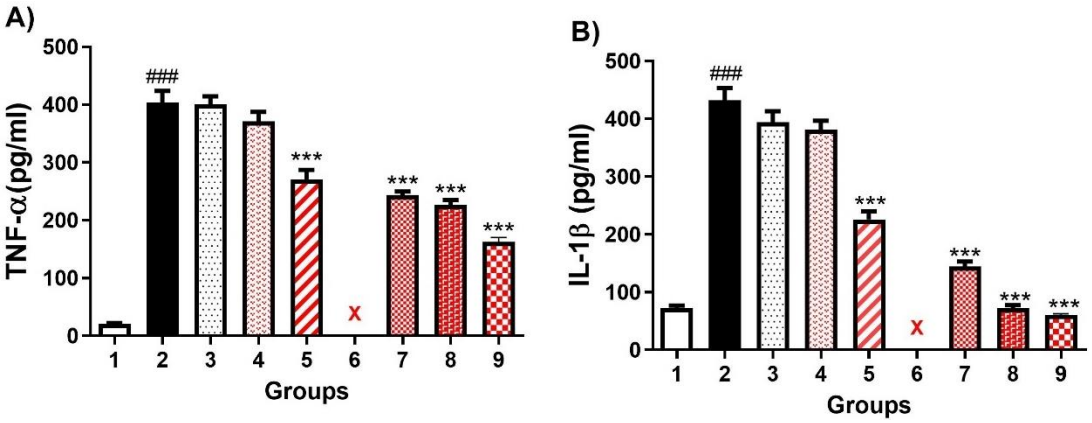

Figure 6.4. Effect of MTX-based formulations on levels of TNF-α (A) and IL-1β (B) in the serum.

1) Normal control; 2) Arthritic control; 3) 0.5% CMC; 4) Blank-SLNs; 5) standard MTX (0.1 mg/kg); 6) standard MTX (1 mg/kg); 7) MTX-SLNs (equivalent to 1 mg/kg of MTX); 8) HA-MTX-SLNs (equivalent to 0.1 mg/kg of MTX); 9) HA-MTX-SLNs (equivalent to 1 mg/kg of MTX). The values are expressed as mean ± SD. ###p<0.001 was considered significant when compared to the normal control group, whereas ***p<0.001 were considered significant when compared to the arthritic control group.

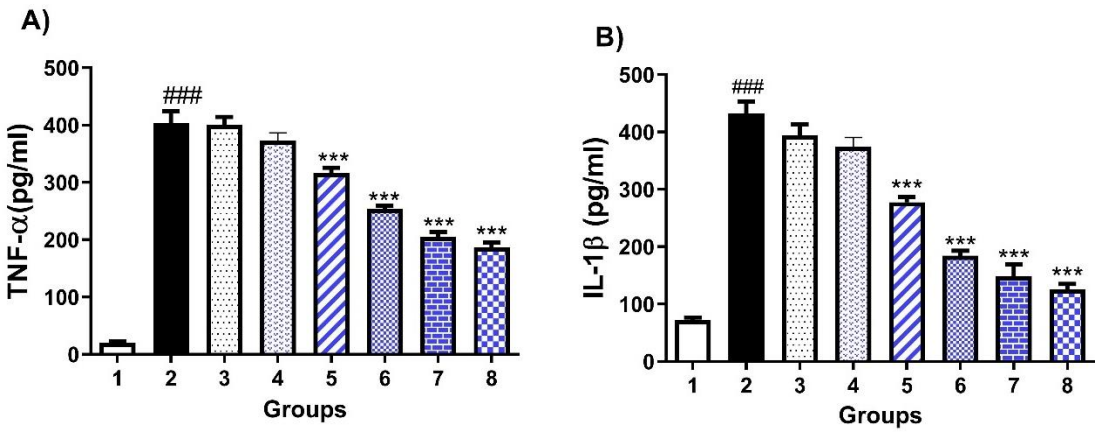

Figure 6.5. Effect of QE-based formulations on levels of TNF-α (A) and IL-1β (B) in the serum.
1) Normal control; 2) Arthritic control; 3) 0.5% CMC; 4) Blank-SLNs; 5) QE standard (30 mg/kg); 6) QE-SLNs (equivalent to 30 mg/kg of QE); 7) HA-QE-SLNs (equivalent to 15 mg/kg of QE); 8) HA-QE-SLNs (equivalent to 30 mg/kg of QE). The values are expressed as mean ± SD. $^{###}p< 0.001$ was considered significant when compared to the normal control group, whereas $^{***}p<0.001$ was considered significant when compared to the arthritic control group.

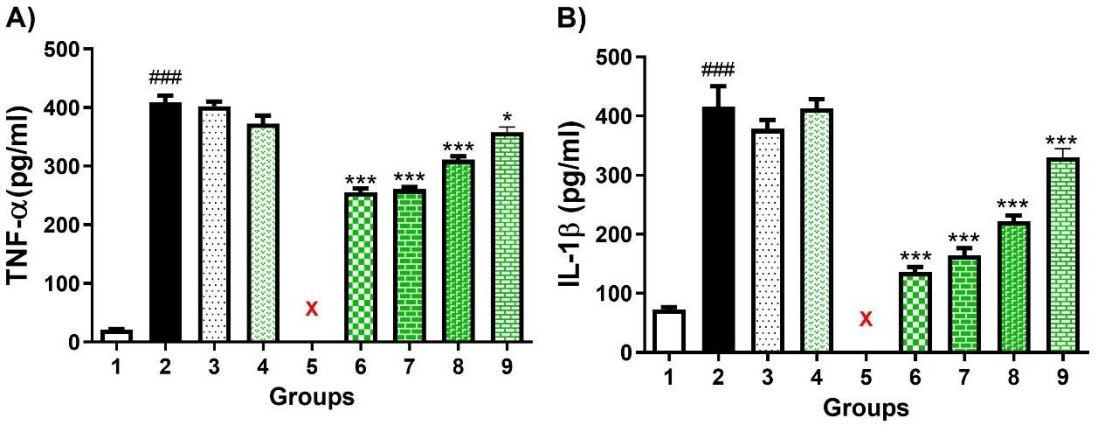

Figure 6.6. Effect of the combination therapy on levels of TNF-α (A) and IL-1β (B) in the serum.
1) Normal control; 2) Arthritic control; 3) 0.5% CMC; 4) Blank-SLNs; 5) MQ (1 mg/kg of MTX + 30 mg/kg of QE); 6) SMQ-I (equivalent to 1 mg/kg of MTX + 30 mg/kg of QE); 7) SMQ-II (equivalent to 1 mg/kg of MTX + 15 mg/kg of QE); 8) SMQ-III (equivalent to 0.1 mg/kg of MTX + 30 mg/kg of QE); 9) SMQ-IV (equivalent to 0.1 mg/kg of MTX + 15 mg/kg of QE). The values are expressed as mean ± SD. $^{###}p<0.001$ was considered significant when compared to the normal control group, whereas $^{*}p<0.05$ and $^{***}p<0.001$ were considered significant when compared to the arthritic control group.

Oxidative Markers: Antioxidants such as Glutathione peroxidase (GPx), and Superoxide dismutase (SOD) activity were evaluated in the liver kidney and spleen of the animals after 21 days.

It was observed (figure 6.7–6.9) that GPX activity was maximum in animals belonging to normal control group and minimum in animals from arthritic control and blank-SLNs group. Amongst all the other treatment groups HA-QE-SLNs were able to retain GPx activity liver and spleen. Similarly, even SMQ-I group (HA-MTX-SLNs + HA+QE+SLNs) at their maximum dose were able to increase the GPx activity in liver and spleen. On the contrary no significant enhancement in GPx activity in any of the three organs were observed in MTX, MTX-SLNs and HA-MTX-SLNs treatment groups.

Similar trend was observed in SOD activity as well (figure 24), wherein arthritic control and blank SLNs treatment groups exhibited lowest SOD activity. While HA-QE-SLNs were able to retain SOD activity near to normal in the liver and spleen. Combination therapy (SMQ-1) showed a better response than HA-MTX-SLNs treatment groups. Quercetin being an antioxidant was expected to show better response in maintaining activity of both GPx and SOD in the vital organs when compared to MTX.

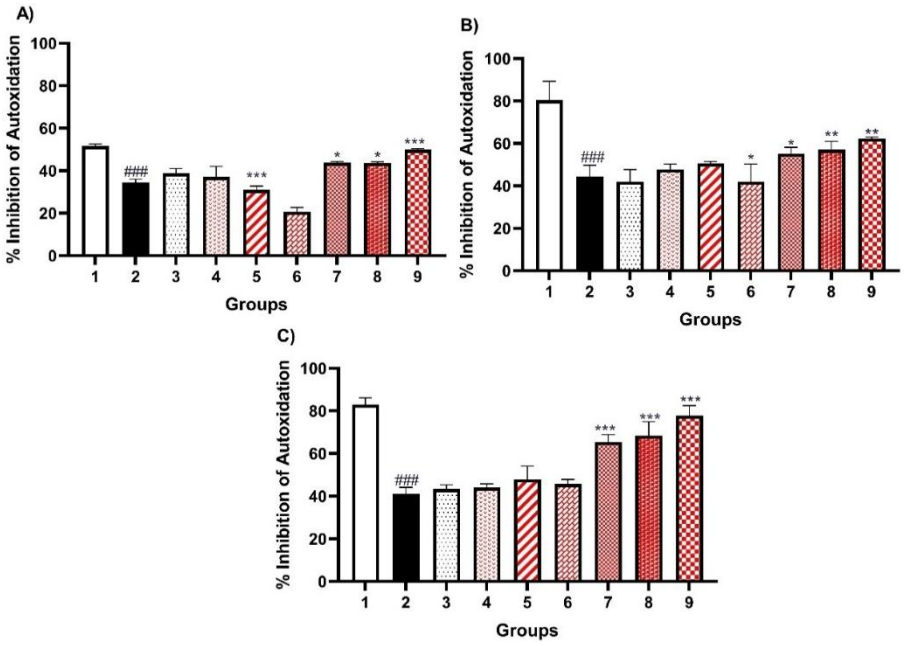

Figure 6.7. Effect of MTX-based formulations on SOD activity in liver (A), kidney (B), and spleen (C).

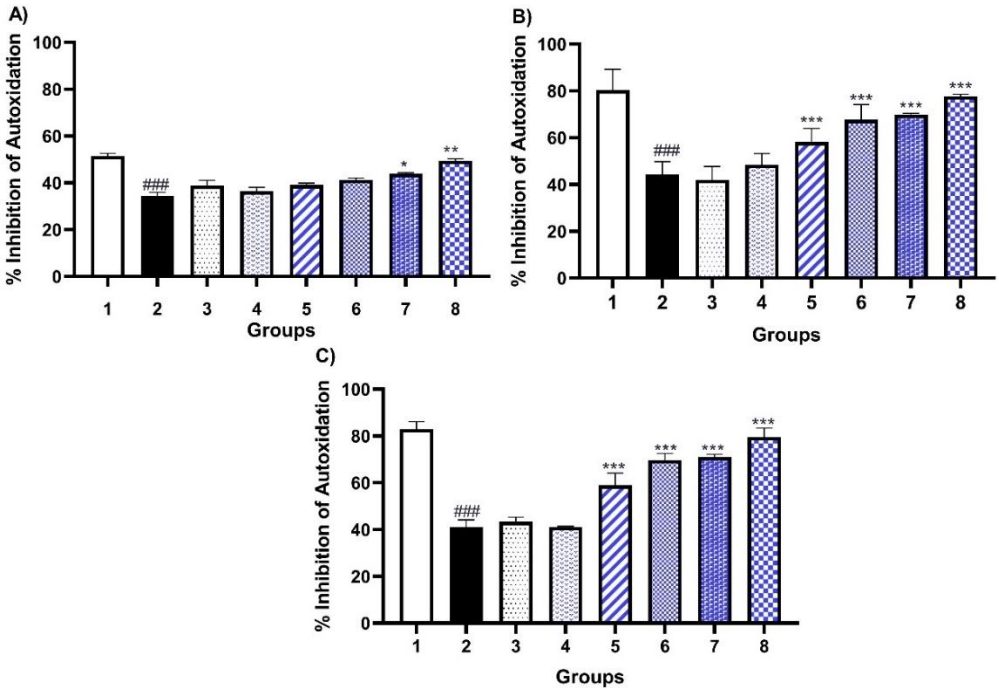

Figure 6.8. Effect of QE-based formulations on SOD activity in liver (A), kidney (B), and spleen (C).

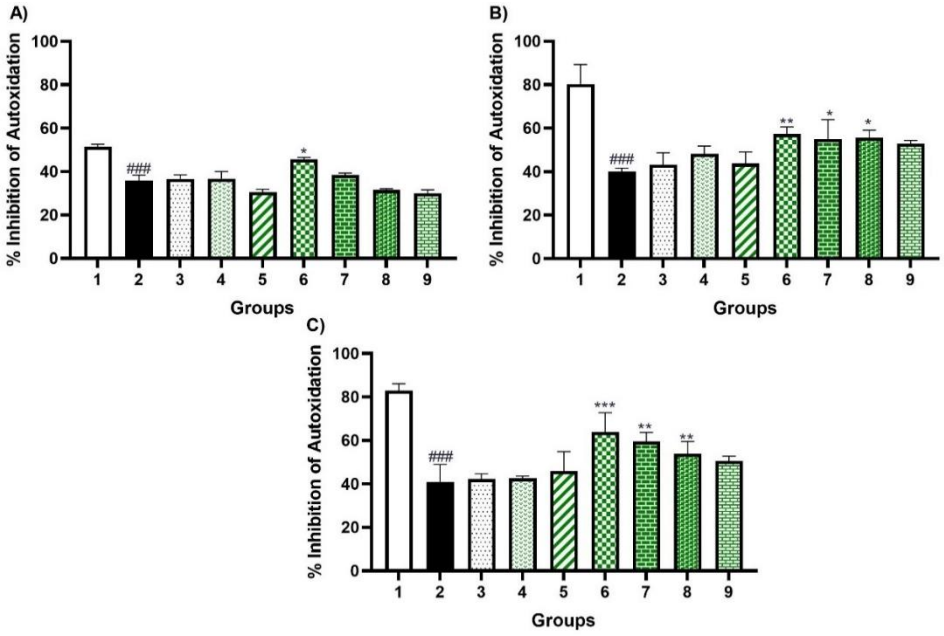

Figure 6.9. Effect of the combination therapy on SOD activity in liver (A), kidney (B), and spleen (C).

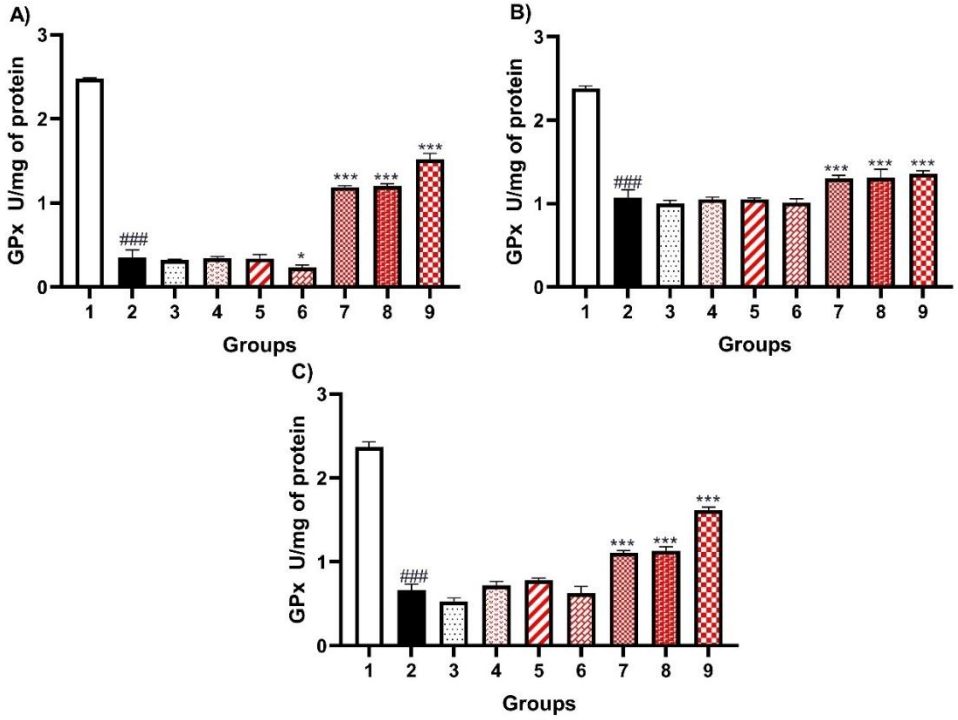

Figure 6.10. Effect of MTX-based formulations on GPx activity in liver (A), kidney (B), and spleen (C).

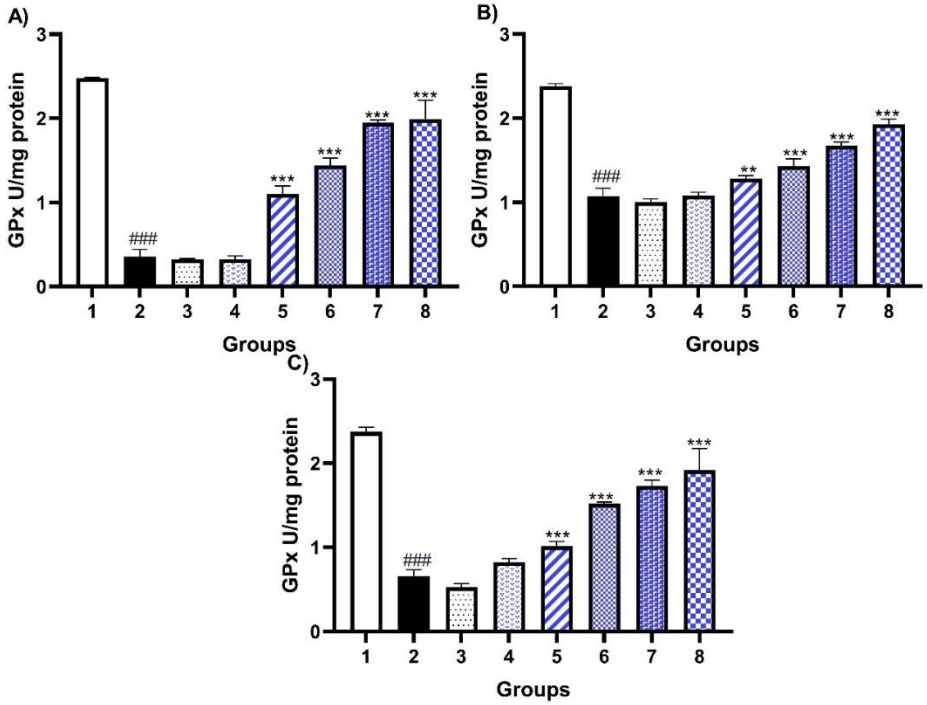

Figure 6.11. Effect of QE-based formulations on GPx activity in liver (A), kidney (B), and spleen (C).

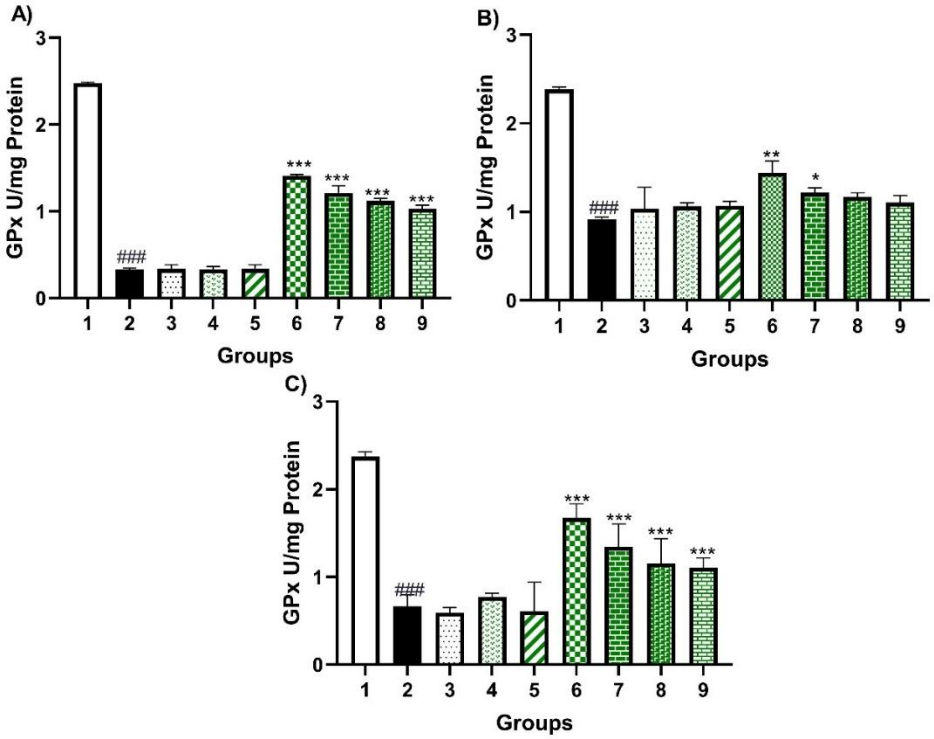

Figure 6.12. Effect of the combination therapy on GPx activity in liver (A), kidney (B), and spleen (C).

Radiographic analysis:

Radiographic evaluation of the hind paws of animals from all the groups was carried out using a Wipro GE Dx300 X-ray unit at, Ovi Diagnostics, Mumbai, India. Lateral radiographs were obtained on day 21. Figure 25, represent the radiographic images of CFA induced animals treated as mentioned in table 6. Radiographic analysis denotes that soft tissue swelling, reduced bone density and narrowing of joint space. Was observed in all CFA induced groups except for normal control. Maximum swelling, reduced bone density and narrowing of joint space was seen in animal belonging to arthritic control group. Standard drug treatment (MTX, QE and combination of both) did show moderate soft tissue swelling however, uncoated drug loaded SLNs also showed soft tissue swelling. HA coated drug loaded SLNs (HA-MTX-SLNs and HA-QE-SLNs) at the maximum dose showed minimum swelling and other parameters too. Combination therapy did show improvement in the soft tissue swelling and other parameters but were not significant when compared to the monotherapy

Figure 6.16. Radiographic images of the paw of animals from MTX treatment groups.

1) Normal control; 2) Arthritic control; 3) 0.5% CMC; 4) Blank-SLNs; 5) standard MTX (0.1 mg/kg); 6) standard MTX (1 mg/kg); 7) MTX-SLNs (equivalent to 1 mg/kg of MTX); 8) HA-MTX-SLNs (equivalent to 0.1 mg/kg of MTX); 9) HA-MTX-SLNs (equivalent to 1 mg/kg of MTX).

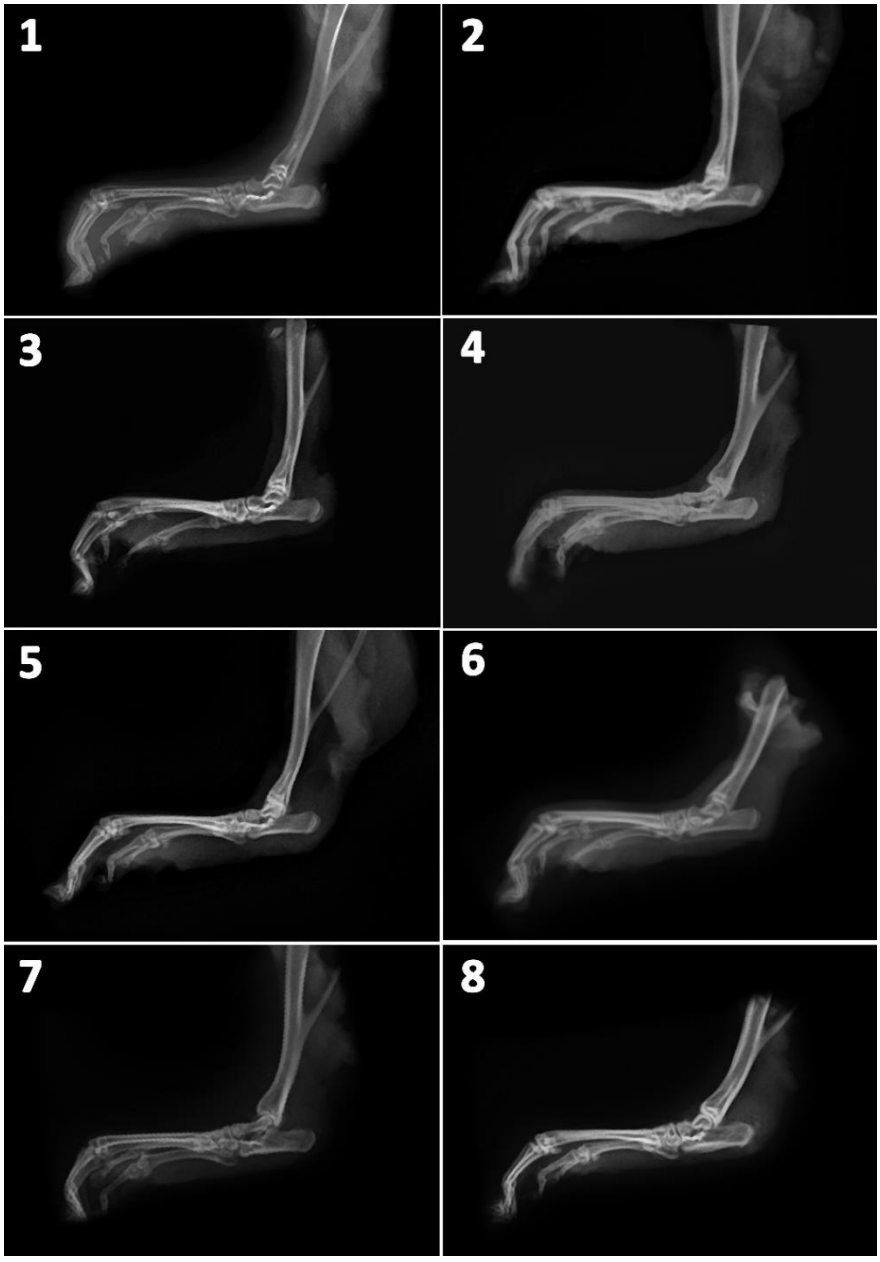

Figure 6.17. Radiographic images of the paw of animals from QE treatment groups.

1) Normal control; 2) Arthritic control; 3) 0.5% CMC; 4) Blank-SLNs; 5) QE standard (30 mg/kg); 6) QE-SLNs (equivalent to 30 mg/kg of QE); 7) HA-QE-SLNs (equivalent to 15 mg/kg of QE); 8) HA-QE-SLNs (equivalent to 30 mg/kg of QE).

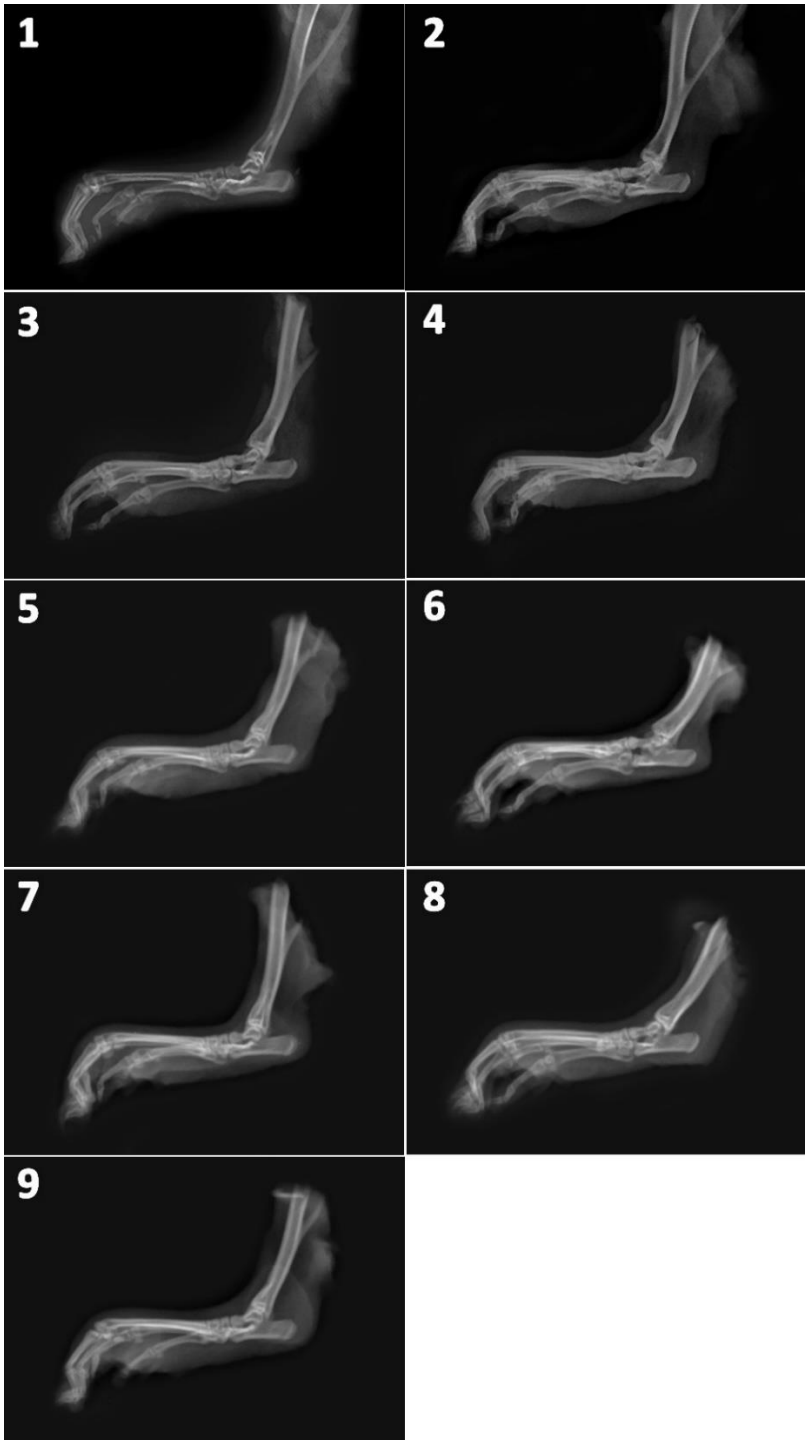

Figure 6.18. Radiographic images of the paw of animals from combination therapy treatment groups. 1) Normal control; 2) Arthritic control; 3) 0.5% CMC; 4) Blank-SLNs; 5) MQ (1 mg/kg of MTX + 30 mg/kg of QE); 6) SMQ-I (equivalent to 1 mg/kg of MTX + 30 mg/kg of QE); 7) SMQ-II (equivalent to 1 mg/kg of MTX + 15 mg/kg of QE); 8) SMQ-III (equivalent to 0.1 mg/kg of MTX + 30 mg/kg of QE); 9) SMQ-IV (equivalent to 0.1 mg/kg of MTX + 15 mg/kg of QE).

CHAPTER 7

SUMMARY CONCLUSION AND FUTURE PROSPECTS

The project was carried out to reduce the toxicity associated with methotrexate (MTX) incorporating it into a safe biocompatible nanocarrier as well as administering it in combination with an anti-oxidant (quercetin QE). The study first began with carrying out the compatibility studies between MTX and QE using various analytical techniques (FTIR, DSC, XRD, HPLC) and molecular simulation which showed no physicochemical interaction, indicating the combination can be taken forward for combination therapy. Then based on the solubility of the drug in different lipids and surfactants the glyceryl monostearate and pluronic f-68 were chosen to prepare the solid lipid nanoparticles by high-speed homogenization method. Quality-based Design approaches were used for the development and optimization of the SLNs. Hyaluronic acid (HA) was used as a coating agent for targeted drug delivery. The synthesized SLNs were characterized for their size, PDI, Surface charge, morphology, sustained drug release, crystalline nature. Both MTX-loaded SLNs and QE-loaded SLNs were spherical and raging between 200–350 nm in size and PDI<0.35. Both MTX-loaded SLNs and QE-loaded SLNs showed 70–80 % entrapment efficiency, sustained drug release, and crystalline nature. The HA-coated formulations were assessed for acute oral toxicity and pharmacokinetic parameters, which delineated increased bioavailability and safety of the drug by using SLNs as drug carriers. The formulations were then evaluated for their efficacy incomplete Freund's adjuvant (CFA) induced arthritis: The data demonstrated enhanced efficacy with no toxicity of the drugs delivered using SLNs. Co-administration of the HA-MTX-SLNs and HA-QE-SLNs did show therapeutic efficacy but when compared to the individual drugs the efficacy was not significant. Hence, indicating that though both the drug did not show any physicochemical interaction, they show an antagonistic effect which could be due to the possibility of an interaction occurring between the two at a physiological level. Therefore, this finding should be dealt with molecular docking to find out the point of interaction occurring between the two molecules. The future scope also lies in exploring other antioxidants that can be used in combination with MTX which can significantly enhance the therapeutic efficiency and reduce drug toxicity. One should also try to develop an SLN drug carrier that can be coloaded with two drugs for better patient compliance.

Hence we can conclude that SLNs are safe nanoparticle-based drug carriers that provide sustained drug release and provide enhanced drug bioavailability. SLNs remarkably reduced toxicity associated with MTX while targeted drug delivery by HA were significantly enhanced the therapeutic efficiency of both the drugs MTX and QE. HA-MTX-SLNs should be further investigated to be introduced into the market as a transformed treatment for RA.

CHAPTER 8
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